Regulation and function of SIRT1 in rheumatoid arthritis synovial fibroblasts

Engler, Anna ; Tange, Clare ; Frank-Bertoncelj, Mojca ; Gay, Renate E ; Gay, Steffen ; Ospelt, Caroline

Abstract: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation and destruction of synovial joints. The function of sirtuin (SIRT)1 in RA is inconclusive. In human synovial cells, SIRT1 was shown to promote cytokine production and apoptosis resistance. However, deletion of SIRT1 aggravated inflammatory arthritis in mice and increased production of pro-inflammatory cytokines in murine macrophages. In the current study, we investigated the regulation, expression, and function of SIRT1 in RA, in particular its role in adhesion and proliferation of human RA synovial fibroblasts (RASF). We found that expression of SIRT1 was increased in vivo in synovial tissues of RA smokers and in vitro by stimulation of RASF with TNF , but decreased upon treatment with cigarette smoke extract. Synovial tissues of RA smokers showed higher leukocytic infiltration that positively correlated with enhanced levels of SIRT1. Global transcriptome analysis revealed that SIRT1 modulates expression of genes involved in the regulation of inflammatory response and cell adhesion. In functional studies, silencing of SIRT1 reduced proliferation and leukocytic adhesion to RASF but showed inconsistent results in the regulation of adhesion to plastic. In conclusion, SIRT1 modulates the proliferative and potentially also adhesive properties of RASF and can therefore promote progression of RA. KEY MESSAGES: SIRT1 is upregulated by TNF but decreased upon CSE treatment of RASF. Upregulation of SIRT1 in RA smokers correlates with increased leukocytic infiltration. SIRT1 modulates expression of genes regulating cell adhesion and inflammation. SIRT1 regulates proliferation of RASF.

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Regulation and function of SIRT1 in rheumatoid arthritis synovial fibroblasts

Anna Engler, Msc [1,2], Clare Tange[1], PhD, Mojca Frank-Bertoncelj, MD, PhD [1], Renate E. Gay, MD[1], Steffen Gay, MD[1], Caroline Ospelt, MD, PhD [1]

[1] Center of Experimental Rheumatology, University Hospital Zurich, Gloriastrasse 25, 8091 Zurich & Wagistrasse 14, 8952 Schlieren, Switzerland

[2] Zurich Center of Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Corresponding author:
Caroline Ospelt, MD, PhD
Center of Experimental Rheumatology,
Bio-Technopark Schlieren
University Hospital Zurich

Wagistrasse 14, 8952 Schlieren, Switzerland
Tel. +41 44 556 3171
FAX +41 44 556 3444
Email caroline.ospelt@usz.ch

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation and destruction of synovial joints. The function of sirtuin (SIRT)1 in RA is inconclusive. In human synovial cells SIRT1 was shown to promote cytokine production and apoptosis resistance. However, deletion of SIRT1 aggravated inflammatory arthritis in mice and increased production of pro-inflammatory cytokines in murine macrophages. In the current study we investigated the regulation, expression and function of SIRT1 in RA, in particular its role in adhesion and proliferation of human RA synovial fibroblasts (RASF). We found that expression of SIRT1 was increased *in vivo* in synovial tissues of RA smokers and *in vitro* by stimulation of RASF with TNFα, but decreased upon treatment with cigarette smoke extract. Synovial tissues of RA smokers showed higher leukocytic infiltration that positively correlated with enhanced levels of SIRT1. Global transcriptome analysis revealed that SIRT1 modulates expression of genes involved in the regulation of inflammatory response and cell adhesion. In functional studies, silencing of SIRT1 reduced proliferation and leukocytic adhesion to RASF, but showed inconsistent results in the regulation of adhesion to plastic. In conclusion, SIRT1 modulates the proliferative and potentially also adhesive properties of RASF and can therefore promote progression of RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease primarily affecting synovial joints and leading to a progressive destruction of cartilage and bone. A combination of genetic predisposition, immunological deregulation and environmental factors contribute to its pathogenesis [1]. The most recognized environmental risk factor for the development and severity of RA is cigarette smoking [2-4]. RA synovium is characterized by synovial hyperplasia and infiltration of inflammatory cells together with high production of pro-inflammatory cytokines such as tumor necrosis alpha (TNFα) [5]. Thus, inhibition of inflammatory processes is the main strategy in the treatment of RA.

SIRTs are class III histone deacetylases that regulate the activity of histones and non-histone proteins by posttranslational modifications [6]. Initially, SIRTs were identified as regulators of cell metabolism, survival, ageing and inflammation [7]. Recent studies demonstrated that SIRTs are involved in the regulation of signaling pathways induced by environmental stress factors, such as cigarette smoking. Exposure to cigarette smoke reduced the expression of SIRT1 in human and rat lungs as well as in human endothelial, epithelial and monocyte-macrophage cell lines [8-10].

The role of SIRT1 in cancer and inflammation, in particular arthritis is controversial. Expression of SIRT1 was shown to be increased in the synovium of RA patients [13]. Down regulation of SIRT1 or inhibition of sirtuin activities in synovial cells of RA patients was found to reduce the expression of inflammatory mediators [13, 14], and overexpression of SIRT1 increased production of pro-inflammatory cytokines in rheumatoid arthritis synovial fibroblasts (RASF) and monocytes from healthy persons [13]. In contrast, levels of SIRT1 were diminished in joint tissues of mice with collagen-induced arthritis [15]. Moreover, deletion of SIRT1 aggravated inflammatory arthritis in serum transfer arthritis model and increased production of pro-inflammatory cytokines in macrophages [16].

In the current study we further analysed the role of SIRT1 in RA. To elucidate the function of SIRT1 in RA synovium we used a next generation sequencing approach to identify specific targets and biological processes regulated by SIRT1 in RASF.

Material and Methods

Patient samples and cell culture

Synovial tissues were obtained from RA patients undergoing joint replacement surgery at the Schulthess Clinic Zurich after obtaining informed written consent according to the principles of the 1964 Declaration of Helsinki. For experiments with human tissues, ethical approval was obtained at the Swiss Ethical commission. RA patients fulfilled the American College of Rheumatology criteria for the classification of RA [17]. RASF were isolated and cultured as described previously [18]. Cells between passages 4 to 8 were used.

CSE preparation and stimulation assays

Cigarette smoke extract (CSE) was prepared using a modification of the method described by Vassallo et al [19]. 100% CSE was prepared by bubbling of the smoke of one cigarette into 10 ml of cell culture medium at a rate of one cigarette per 7 minutes. CSE was sterile filtered through 0.2 μm filter and diluted to 5% CSE with cell culture medium. The pH of 5% CSE and of the control cell culture medium was equal and remained unaltered during the cultivation. The cigarettes used contained 10 mg tar, 0.8 mg nicotine and 10 mg carbon monoxide (Marlboro Red). RASF were stimulated with 5% CSE or 10 ng/ml TNFα (R&D systems) for 24 hours.

Overexpression and gene silencing

RASF were transfected using the Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Amaxa) according to the manufacturer's instructions. In silencing experiments, 100 μM of validated siRNA for SIRT1 or control siRNA (Invitrogen) were used. For overexpression of SIRT1, 1 μg of SIRT1 wild-type or empty pcDNA3.1(-) (mock) expression vectors were used

as previously described [13]. Silencing and overexpression of SIRT1 were verified by Western blot analysis or real-time PCR (Supplementary Fig.1).

RNA isolation and quantitative real-time PCR

RNA was isolated, reverse transcribed and gene expression quantified as previously described [20]. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as an endogenous control. The primer sequences used are given in the Supplementary table S1.

Next generation sequencing and functional annotation analysis

For transcriptome analysis the expression of SIRT1 was down or up regulated in RASF (n=2) for 72h as described above. RNA was isolated using RNeasy Plus Micro Kit (Qiagen) followed by treatment with RNase-free DNase (Invitrogen). RNA was cleaned-up using RNeasy Mini Kit (Qiagen). The library constructs were produced using TruSeq Stranded mRNA Sample Preparation Kit according to Illumina TruSeq® Stranded mRNA protocol. Libraries were pooled and further used for cluster generation using TruSeq SR Cluster Kit v3-cBot-HS reagents and sequenced with TruSeq SBS Kit v3-HS reagents on Illumina HiSeq 2500 in the high output mode. For pathway enrichment analysis only coding transcripts that were found to be altered by silencing as well as by overexpression of SIRT1 (cut-off log2 ratio > +/-0.4) and had a signal intensity of >10 were taken into account. Functional annotation clustering was done using the Database for Annotation, Visualization and Integrated Discover (DAVID) [21, 22]. Gene Ontology (GO) terms corresponding to the biological processes (BP-FAT) were used to group the genes. The significance of the gene-term enrichment was determined by Fischer`s exact test and p values ≤ 0.05 were considered strongly enriched.

Proliferation and adhesion assays

Proliferation and adhesion of RASF were measured in the xCELLigence Real-Time Cell Analyzer (RTCA) DP system (Bucher Biotec AG). For proliferation assay, RASF were transfected with SIRT1 siRNA or control siRNA as described above and 2500 cells were seeded in each well. Medium was changed 24h after transfection. The rate of proliferation was determined by calculating the slope of the cell index (CI) curve in the interval between 40 and 85h after transfection, since the best efficacy of SIRT1 silencing was observed 48 – 96h after transfection. To investigate the effect of SIRT1 on adhesion, RASF were transfected for 72h with SIRT1 siRNA or control siRNA as described above. Cells were detached with accutase and 2500 cells were seeded in each well. The rate of adhesion was determined by calculating the slope of the CI curve between 0 and 60min after seeding of cells.

Leukocyte adhesion assay

Leukocyte adhesion to RASF was performed based on the protocol by Tessier et al.[23]. Peripheral blood mononuclear cells (PBMCs) were isolated from whole EDTA blood from one healthy donor by Ficoll paque plus (GE Healthcare). RASF were transfected with SIRT1 or scrambled siRNA for 72h before $1x10^6$ PBMCs were added to 3×10^4 RASF for 1 hour. The wells were washed 3 times with PBS and the adherent cells were stained with 0.01% methylene blue, 0.05% crystal violet, 2% methanol, 3% acetic acid and 95% distilled H_2O. PBMCs were counted in 4 visual fields (100x magnification) per condition.

Immunohistochemistry

Formalin-fixed, paraffin-embedded synovial tissues (n=23) were deparaffinised and pretreated with 10 mM citrate buffer (pH=6) for antigen unmasking. After washing with ddH_2O, blocking of endogenous peroxidase activity (3% H_2O_2) and of non-specific binding (5% goat serum in antibody diluent (Dako)), slides were incubated with rabbit IgG (Jackson ImmunoResearch) or rabbit anti-SIRT1 antibody (E104, Abcam, #ab32441, 1:40 in antibody

diluent). After washing, the slides were incubated with biotinylated goat-anti-rabbit antibodies (Jackson ImmunoResearch, 1:500 in antibody diluent/5% human serum) and the Dako EnVision+ System- for horseradish peroxidase (HRP). SIRT1-positive cells were visualized using DAB (3.3'-Diaminobenzidin). Nuclei were counterstained with hematoxylin. The intensity of the SIRT1 staining in synovial tissues was evaluated by 2 independent observers, using a gradual scoring scale from 0 (no staining) to 4 (strong staining). For the assessment of synovitis in synovial tissues, thickness of lining cell layer, cellular density of synovial stroma and leukocytic infiltrate were semiquantitatively evaluated in hematoxilin/eosin stained tissues as described by Krenn et al. [24]. Patient's characteristics are shown in Table 1. In slides stained for SIRT1, stromal characteristics could not be evaluated and thus the synovitis score comprised thickness of lining layer and infiltration only.

Table 1: Characteristics of the patients.

	RA smoker n=22	**RA nonsmoker n=27**
Age (years, mean (range))	55 (33-80)	61 (31-78)
Sex (female/male)	17/5	22/5
Disease duration (years, mean (range))	21 (4-48); 1 ND	20 (4-48); 3 ND
number of cigarettes per day (mean (range))	15 (3-40)	
RF positiv/negative	14/3; 5 ND	14/3; 10 ND
Medications		
DMARDs	14	15
NSAIDs	13	13
steroids	12	11
biologics	2	10

ND = not determined; DMARDs = disease modifying anti-rheumatic drugs; NSAIDs = non steroidal anti-inflammatory drugs

Immunoblotting

Whole cell lysates were prepared by lysing the cells in Laemmli buffer. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to

nitrocellulose membranes. Membranes were blocked with 5% milk in TBS-T and incubated over night at 4°C with mouse anti-proliferating cell nuclear antigen (PCNA) (PC10, Novus Biologicals #NB500-106, 1:1000), mouse anti-tubulin (DM1A, Abcam, # ab7291, 1:10'000), rabbit anti-SIRT1 (E104, Abcam, #ab32441, 1:500) or mouse anti-Vimentin antibodies (Abcam, #ab7752, 1:1000) in 5% milk.HRP-labeled species specific secondary antibodies (Jackson ImmunoResearch, 1:10000) and enhanced chemiluminescence (GE Healthcare) were used for visualization.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. Values are presented as mean±SEM. Data were analyzed using t-test or Wilcoxon Signed Rank Test as indicated. Correlation of SIRT1 expression with levels of synovitis or infiltration was investigated using Spearman correlation. P-values <0.05 were considered significant.

Results

Regulation of SIRT1 expression in RASF and synovial tissues

Smoking and TNFα are critical factors in the pathogenesis of RA. To investigate the regulation of SIRT1 in RASF, we stimulated the cells with 5% CSE or 10 ng/ml TNFα for 24h. Western blot analysis revealed decreased protein levels of SIRT1 in RASF stimulated with CSE (Figure 1a). Opposite to CSE, stimulation of RASF with TNFα increased protein expression of SIRT1 (Figure 1a). Co-stimulation with CSE and TNFα together increased the protein levels of SIRT1 indicating that up regulation of SIRT1 by TNFα is dominant over down regulation of SIRT1 after CSE (Figure 1c). To analyze the effect of smoking on SIRT1 expression *in vivo*, we compared the expression of SIRT1 in synovial tissue sections from RA smokers with RA nonsmokers by immunohistochemistry. Scoring of SIRT1 staining intensities revealed increased expression of SIRT1 in synovial tissues of RA smokers when compared to RA nonsmokers (Figures 1c, d).

When expression of SIRT1 in synovial tissues was compared to the degree of synovitis a positive correlation ($p=0.008$) was observed (Figure 2a). By separately analyzing the two components of the synovitis score, namely leukocytic infiltration and thickening of the synovial lining layer, a significant positive correlation between the expression of SIRT1 and leukocytic infiltration was seen ($p=0.01$), while no correlation was found between SIRT1 levels and synovial thickening (Figure 2a). Assessment of synovitis in smoking and nonsmoking RA patients showed that smoking RA patients had significantly higher synovitis score and more inflammatory cells infiltrating the synovial tissue compared to nonsmoking RA patients. Moreover, the density of synovial stroma was increased in smoking RA patients (Figure 2b).

Targets and biological processes regulated by SIRT1 in RASF

To investigate the effect of increased SIRT1 expression in RA synovial tissues and RASF, as shown previously by Niederer et al [13], and the particular high levels of SIRT1 in RA smokers observed in this study, a next generation sequencing approach was applied. The expression of SIRT1 was decreased by SIRT1 silencing or increased by SIRT1 overexpression and changes in gene expression were measured by poly-A-enriched transcriptome sequencing. 46 genes were up regulated by SIRT1 overexpression and down regulated by SIRT1 silencing, whereas 52 genes were down regulated after SIRT1 overexpression and up regulated in SIRT1 silenced RASF (Figure 3, Supplementary table S2). To identify the biological processes affected, we grouped the genes into specific biological processes using the GO annotation program DAVID. After grouping the genes that were up regulated by SIRT1 overexpression and down regulated by SIRT1 silencing we found that the top 5 biological processes with the highest numbers of enriched genes are 'response to organic substance', 'defense response', 'cell adhesion', 'biological adhesion' and 'inflammatory response' (Supplementary table S2). Grouping of genes that were down regulated by SIRT1 overexpression and up regulated by SIRT1 silencing revealed that the top 3 enriched biological processes are 'response to wounding', 'defense response' and 'inflammatory response' (Supplementary table S3). Taken together, modifications of SIRT1 levels induced strongest changes in the expression of genes involved in the regulation of inflammation and cell adhesion.

Verification of SIRT1 targets that might play an important role in the pathogenesis of RA in more patients confirmed increased expression of Cadherin 4 (CDH4) by silencing of SIRT1 (Figure 4a), whereas levels of Chemokine (C-X3-C motif) ligand 1 (CX3CL1) and of JunB proto-oncogene (JunB) were significantly up regulated by overexpression of SIRT1 (Figures 4b, c).

Effect of SIRT1 on adhesion and proliferation of RASF

Even though not reaching statistical significance (p=0.054), adhesion was found to be one of the top enriched biological processes in the functional annotation analysis of SIRT1 target genes. Therefore we investigated the effects of SIRT1 silencing on the regulation of cell adhesion. Expression of SIRT1 in RASF was reduced by silencing of SIRT1 with siRNA, and cell behavior was monitored using the xCELLigence RTCA system. Strongest changes in adhesion of RASF were detected in the first 60 minutes after seeding. Therefore the slope of the curve between 0 and 60 min was calculated and used as a parameter for changes in cell adhesion. While in 3 out of 5 patients adhesion was stronger in SIRT1 siRNA than control siRNA transfected cells, in two patients this effect of SIRT1 silencing on adhesion was not seen (Figures 5a,b). Taking all patients together no significance was reached (Figure 5c).

The chemokine CX3CL1 and the receptor stabilin-2 (STAB2) were listed in the adhesion pathways as well as in inflammatory pathways ('defense response' and 'regulation of inflammatory response'). Both of these genes were implicated in the adhesion of leukocytes to endothelial cells [25, 26]. Therefore we incubated PBMCs with RASF that were silenced for SIRT1 and control transfected RASF, respectively, for 1 hour. After washing, significantly less PBMCs were attached to SIRT1 silenced RASF than to the control transfected RASF (Figure 5d).

SIRT1 was also shown to regulate cell proliferation [27-32]. Altered proliferation of synovial cells was suggested to contribute to the synovial hyperplasia in RA [33]. To study the effect of SIRT1 on proliferation, RASF were seeded immediately after transfection with SIRT1 siRNA and the cell behavior was monitored in real-time over a period of 160 hours using the xCELLigence RTCA system. Proliferation rate of RASF transfected with SIRT1 siRNA was significantly lower than in control transfected cells 40 – 85 hours after transfection (Figure 6b). After around a week of growth the cell index reached the same index in SIRT1 silenced RASF as in control transfected cells indicating that SIRT1 silencing specifically affected

proliferation and not the apoptosis where the cell index would constantly decrease over time (Figure 6a). To confirm an effect of SIRT1 on proliferation we measured levels of PCNA 72h after silencing of SIRT1. PCNA levels were significantly lower in SIRT1 siRNA transfected cells compared to control transfected RASF (Figure 6c).

Discussion

In the current study we investigated the regulation and function of SIRT1 in RA synovium. We found that expression of SIRT1 *in vitro* is differentially regulated by TNFα and CSE exposure in RASF. Expression of SIRT1 protein *in vitro* was down regulated by stimulation of RASF with CSE, whereas treatment with TNFα increased protein levels of SIRT1. At the mRNA level we previously found that the expression of SIRT1 transcript is not affected by stimulation with CSE [34] whereas TNFα stimulation up regulated the levels of SIRT1 mRNA [13]. We studied the regulation of SIRT1 protein *in vivo* and found that SIRT1 levels were up regulated in the synovial tissues of RA smokers and correlated positively with increased leukocytic infiltration. Previously, it was shown that smokers respond less to therapies with disease modifying anti-rheumatic drugs and had higher levels of the chemokine MIP-1α [35]. The same study showed that CSE induces chemotaxis *in vitro*. Thus, smoking could enhance leukocytic infiltration in synovial tissues. This can lead to higher levels of TNFα, which up regulates the expression of SIRT1 in the joints of RA smokers. Indeed, smoking was shown to increase the levels of TNFα in the serum [36]. Moreover, release of TNFα by stimulated T lymphocytes was significantly higher in RA patients who smoked than in those who never smoked [37]. These results are in line with clinical studies showing that RA smokers show a poor response to anti-TNF treatment and RA heavy smokers have the poorest drug survival [38]. At the cellular level, exposure to cigarette smoke was also shown to increase the production of TNFα in inflammatory cells *in vitro* [39-41]. However, as reported previously we could not detect TNFα expression in unstimulated or CSE treated RASF [34]. Since cigarette smoke does not induce TNFα in RASF, the expression of SIRT1 in RASF *in vitro* is regulated by CSE only, whereas *in vivo* increased levels of TNFα in the synovium of RA patients could overlap the effect of smoking resulting in up regulation of SIRT1 levels. Accordingly levels of SIRT1 were increased in RASF stimulated with TNFα

and CSE in combination, which points to a stronger effect of TNFα than CSE in the regulation of SIRT1.

SIRT1 is a multifunctional protein that regulates a variety of cellular processes including cell survival, metabolism, ageing, inflammation and cancerogenesis in different cell types and tissues [7, 42]. To investigate the target genes and biological processes regulated by SIRT1 in RASF, we silenced or overexpressed SIRT1 and found that modifications of SIRT1 levels induce changes in the expression of genes involved in the regulation of inflammation and cell adhesion. The function of SIRT1 in inflammation is controversial. SIRT1 was shown to regulate many anti-inflammatory processes, but also some pro-inflammatory pathways [43, 44]. The anti-inflammatory effect of SIRT1 and also of SIRT6, which we previously found to be induced by smoking, was shown to be mediated by inhibition of NF-kB-dependent gene expression in other cell types [34, 45, 46]. In RASF, however, we and others could show that SIRT6 does not affect the expression of the NF-kB dependent pro-inflammatory cytokines IL-6 or IL-8, but reduces expression of MMP1 via direct deacetylation of the MMP1 promoter [34, 47]. SIRT1 on the other hand was reported to promote the production of these pro-inflammatory cytokines in RASF and human monocytes [13]. Our results confirm the pro-inflammatory role of SIRT1 in RASF, suggesting that neither SIRT6 nor SIRT1 negatively regulate NF-kB in RASF. A possible regulatory pathway in RASF might be the regulation of AP-1, since we found that SIRT1 enhances the expression of JunB, a component of the AP-1 transcription factor complex, which was shown to regulate RA-associated inflammation [48].

We also investigated the role of SIRT1 in the regulation of cell adhesion. Despite effects of SIRT1 on the expression of genes involved in the regulation of adhesion (*CDH4, CDH1*), we could not detect a consistent effect of SIRT1 silencing on adhesion of RASF to the cell culture dish surface in functional assays. The variability in the adhesion of RASF in response to SIRT1 silencing could be triggered by differences in the genetic background of the patients or the heterogeneity of RA. Contrasting effects of SIRT1 were already reported in the

regulation of cancer cells. The dual role of SIRT1 in promoting or suppressing tumor progression was suggested to correlate with the genetic background of the tumor cells and with the presence or absence of p53 [11, 12]. Interestingly, p53 was shown to be overexpressed in RA synovium and to contribute to the regulation of synoviocyte invasiveness, apoptosis and proliferation [49-51]. Somatic mutations in the *p53* gene were detected in RA synovium and were functionally linked to the regulation of inflammation [52, 53]. Thus, depending on the expression of wild type or mutated form of p53 in RA patients the effect of SIRT1 in RASF could be more or less prominent. Furthermore, the transcription factor p53 was reported to control the transcription of CX3CL1 [54]. Therefore SIRT1-dependent changes of CX3CL1 in RASF might be mediated via p53.

CX3CL1 and STAB2, which were both upregulated by SIRT1, were previously described to increase adhesion of leukocytes to endothelial cells [25, 26]. Their downregulationmight also be responsible for the decreased adhesion of leukocytes to SIRT1 silenced RASF.

Previously we could show that SIRT1 induces apoptosis in RASF [13]. Here, we found that in addition silencing of SIRT1 reduced the proliferation rate of RASF. Similarly, decreased proliferation upon silencing of SIRT1 was reported in human malignant glioma cells, melanoma cells, vascular smooth muscle cells and murine osteoprogenitors [27-30]. However, SIRT1 was also shown to inhibit proliferation in human colon cancer cells and murine gut epithelial cells [31, 32]. Since CX3CL1 was previously reported to increase the proliferation of endothelial cells [55] and we found that CX3CL1 levels are reduced by SIRT1 silencing it is feasible to assume that SIRT1 silencing decreases proliferation of RASF via reduction of CX3CL1 levels.

In summary, our data showing that SIRT1 contributes to the activation of human synovial cells by promoting the pro-inflammatory, proliferative and potentially adhesive properties of RASF suggest an adverse function of SIRT1 in the pathogenesis of RA and are in line with previous reports in human cells [13, 14]. In mice, however, SIRT1 was shown to have the

opposite effects, since deletion of SIRT1 aggravated the inflammatory arthritis in serum transfer arthritis model and increased production of pro-inflammatory cytokines in murine macrophages [16]. Whether these different effects of SIRT1 could be species or cell type specific needs to be further elucidated. Thus, it is important to reconsider the influence of substances that modify SIRT1 activity in all cell types prior their application in murine models or clinical trials.

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Competing interests:

None declared.

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Figure legends

Figure 1: Expression of SIRT1.

a) Stimulation of RASF (n=6) with 5% CSE for 24h decreases the protein levels of SIRT1, but stimulation of RASF (n=10) with 10 ng/ml TNFα for 24h increased the protein levels of SIRT1. b) Co-stimulation of RASF with CSE and TNFα together increased the protein levels of SIRT1 in control transfected RASF. (c) Scoring of SIRT1 staining intensities in synovial tissues of RA non smokers (n=11) and RA smokers (n=12) shows increased SIRT1 levels in the synovium of RA smokers. (d) Representative sections of RA synovial tissues showing higher expression of SIRT1 (brown) in RA smokers as compared to RA non smokers. Insets show the control IgG staining. Nuclei were counterstained with hematoxylin (blue). Original magnification: ×100 (top) and×400 (bottom); scale bar = 100µm.

Figure 2: Synovitis score.

(a) Enhanced expression of SIRT1 correlates with increased synovitis score and leukocytic infiltration in RA synovial tissues. (b) Synovial tissues of RA smokers show higher synovitis score and leukocytic infiltration than the synovial tissues of RA non smokers. *= $p<0.05$ as measured by t-test.

Figure 3: Modification of SIRT1 levels induces global changes in the gene expression in RASF.

Levels of SIRT1 were decreased by SIRT1 silencing or increased by transfection with SIRT1 specific vector in RASF (n=2). Changes in the global gene expression were measured by poly-A-enriched transcriptome sequencing. (a) Genes that were down regulated after overexpression of SIRT1 and up regulated after silencing of SIRT1. (b) Genes that were up regulated by SIRT1 overexpression and down regulated by silencing of SIRT1.

Figure 4: Modification of SIRT1 levels induces changes in the gene expression in RASF.

Levels of SIRT1 were decreased by SIRT1 silencing or increased by transfection with SIRT1 specific vector in RASF (n=4-10). Silencing of SIRT1 significantly increased the expression of CDH4 (a) whereas overexpression of SIRT1 enhanced the levels of CX3CL1 (b) and JunB (c). *$p<0.04$ as determined by Wilcoxon signed rank test.

Figure 5: Effect of SIRT1 silencing of rate of adhesion in RASF.

The expression of SIRT1 was down regulated by transfection of RASF with SIRT1 specific siRNA for 72h. Cells were seeded in E-plates and the rate of adhesion was determined by analysing the slope of the curves in the first 60 minutes after seeding of the cells in real-time using the xCELLigence RTCA system. (a) Silencing of SIRT1 (black line) altered the rate of adhesion in 3 patients as compared to control transfected cells (grey line), whereas no effect of SIRT1 silencing on adhesion was observed in 2 patients (b). Taking all patients together, no significant changes in the rate of adhesion upon SIRT1 silencing were detected (c). (d) Significantly less PBMCs attached to SIRT1 silenced RASF compared to control transfected RASF after 1h of co-incubation. Representative pictures of 1 out of 6 experiments are shown. *$p<0.04$ as determined by Wilcoxon signed rank test.

Figure 6: Silencing of SIRT1 decreases rate of proliferation in RASF.

For silencing of SIRT1 RASF were transfected with SIRT1 siRNA (black line) or control siRNA (grey line) and seeded directly in E-plates. The graphs show dynamics and rate of proliferation in RASF in one representative experiment(a,). The rate of proliferation was monitored in real-time using the xCELLigence RTCA system and was determined by analysing of the slope of the curves between 40 and 85 hours (n=5) (b). PCNA levels were measured after 72h of transfection with siRNA against SIRT1 or scrambled control (n=7) (c). ** = $p<0.01$, ***=$p<0.001$ as measured by t-test.

Supplementary figure 1: Verification of silencing and overexpression of SIRT1.

Expression of SIRT1 was silenced by transfection of RASF with SIRT1 (S1) specific siRNA or control (co) siRNA and up regulated by transfection of RASF with SIRT1 overexpression vector or control vector for 72h. Efficacy of silencing or overexpression was verified by Real-time PCR (a) or Western blot analysis (b). *$p<0.04$ as determined by Wilcoxon signed rank test.